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Lipid Determination in Powdered Human Dentin by Thin-Layer and Gas-Liquid Chromatography

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Recent improvements in lipid assays (thin layer chromatography and gas liquid chromatography instrumentation) motivated reinvestigation of the lipid content in human dentin. The present report describes the technics utilized for the separation and quantitative assay of the various lipid components in dentin, a new method of preparing the dentin material, and evaluation of the errors involved in these methods. Although there have been various reports on lipid composition,[1-7] none of these reports analyzed for all the lipid components that may be presently analyzed for, utilized the latest available technics, or determined the limits of reliability of the analytical data.

Materials and Methods

PREPARATION OF DENTIN.—Teeth were washed immediately after extraction and kept frozen while not in use. A high-speed drill under a continuous jet stream of ice-cold water was used to remove unwanted materials. The teeth were carefully cleaned of enamel and carious portions and the root canals were enlarged so that ultimately only sound dentin remained. The chunks of sound dentin were placed in a blender* that had been previously chilled, and the material was reduced to a fine powder that would pass through a 200- to 250-mesh sieve. These procedures were performed while keeping the material close to 0°C.

To determine the hydrolytic effect, on triglycerides, of high-speed drilling under a continuous jet stream of ice-cold water, this experiment was carried out:

Freshly extracted teeth of all types were left uncleaned, and 120 Gm. was placed in 1,000 mg. of triolein and 100 mg. of cholesterolpalmitate in 500 ml. of acetone. The acetone was then blown off with N_2. The teeth were then cleansed of unwanted materials by use of a high-speed drill under a continuous jet of cold water. Care was taken to collect all of the washings and materials in a 5-liter beaker. The washings, as well as the solids, were then subjected to a continuous ether extraction (72 hours). The ether solution was dryed over $MgSO_4$, filtered, and concentrated to a small volume. The solution was assayed for triolein, cholesterolpalmitate, and oleic and palmitic acid (TLC) by chromatographic technics that are described subsequently. Results indicated that under these experimental conditions, 27 percent of the triolein and 30 percent of the sterol ester were hydrolyzed.

EXTRACTION OF DENTIN LIPIDS.—Two experimental runs were carried out, each with 100-Gm. samples of the powdered dentin. The dentin and 500 ml. of Bloor's solvent (ethanol-ether 4:1)[8] were placed in a 2-liter, round-bottomed flask provided with a condenser and a bubbling tube. Nitrogen was continuously bubbled through the tube to continuously agitate the whole suspension. This procedure was continued at 0° to 1°C for 3 to 4 days. At the end of this time, the material was decanted and the residue was centrifuged and rewashed with an additional 50 ml. of solvent. The solvent portions were combined. The solvent was removed from the solution under vacuum at room temperature. The oily residue was then dissolved in 500 ml. of hexane and kept at —20°C. until ready for use. Aliquots of this stock solution were used for all the assays. The solvent was removed, when necessary, by bubbling with dry, pure nitrogen.

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* Waring Blendor, Waring Products Corp., Winsted, Conn.

The completeness of extraction was determined by mixing 1,000 mg. of triolein with 30 Gm. of dry, powdered dentin and adding 200 ml. of Bloor's solvent. At 6-hour intervals, the solvent was removed and assayed for triolein content. Results indicated that 90.5 percent of the lipid was extracted in the first 6-hour interval. To achieve total extraction, however, a 72-hour period was required.

THIN-LAYER CHROMATOGRAPHY.—The fractionation of the lipids into the major class components was done by preparative thin-layer chromatography (TLC).[9] The TLC plates (20 by 20 cm.) were coated with silica gel* that had been extracted to remove organic impurities. The layer thickness was approximately 275μ. The plates, after brief air drying, were activated by heating in an oven at 105 C. for 2 hours and then cooled in a desiccator. The plates were marked with five lanes, three 5-cm. lanes for sample application and blank and two 2.5-cm. lanes for the reference lipid mixtures. The lipid samples, 2 to 5 mg. in a 5:1 chloroform dilution, were applied to the plates in the appropriate lanes, using a sample streaker.† The development solvent was a mixture of petroleum ether, ethyl ether, and formic acid (80:20:0.8), and the development distance was 125 mm. The lipids separated into six well-defined zones in order of increasing Rf values; i.e., phospholipids, free sterols, free fatty acids, triglycerides, wax esters-sterol esters, and hydrocarbons. The zones were detected by application of iodine vapors on the reference lanes, care being taken to prevent the sample lanes from contact with the vapor. Zones were scraped from the plate, and the silica gel was collected. Material from lane 1 was used for the preparation of the methyl esters of the various lipid constituents. The material from the remaining lane, together with the silica gel from the blank lane, was used for the quantitative determination of the lipid components by a modification of the procedure described by Amenta.[9]

ESTERIFICATION[10] (PREPARATION OF METHYL ESTERS).—Prior to gas-liquid chromatography, the samples were methylated. A weighed sample of lipid (10 to 40 mg.) was added to a 25-ml. flask provided with a short condenser. The sample was dissolved in 5 ml. of petroleum ether, to which 10 ml. of 0.1N potassium methoxide solution was added. Oxygen-free nitrogen was bubbled slowly through the mixture by means of a glass capillary introduced through the condenser, and the mixture was refluxed for 1½ hours. 0.5N methanol solution of sulfuric acid was added through the condenser in slight excess over the amount calculated to neutralize the methoxide. The contents were then cooled and transferred to a separatory funnel, using two 15-ml. portions of petroleum ether to make the transfer. About 20 ml. of water was then added, and the aqueous layer was reextracted with 20 ml. of petroleum ether in a second small separatory funnel. The two ether extracts were combined and washed with several 15-ml. portions of water until the washings were neutral to Congo red paper. The solution was transferred to a small, preweighed flask, and the solvent was removed by warming on a water bath at 45°C. with a gentle stream of nitrogen. After the solvent was removed, the flask was adjusted to constant weight.

GAS-LIQUID CHROMATOGRAPHY.—The methyl esters were isolated by carbon disulfide extraction and injected directly into the gas chromatography column. Gas-liquid chromatography[11] analyses of the methyl esters were performed in a commercial apparatus‡ equipped with flame ionization detector. The column was coiled stainless steel tubing, ⅛ inch O.D. by 8 feet, packed with 10 percent stabilized diethylene glycol succinate (DEGS) on acid-washed A nakrom 60/70 A. The areas under the peaks were determined by an electronic integrator coupled to a digital printer. Areas and percentages thus obtained had good agreement with known values, obtained with authentic standards.

Results

The total weight of the lipid sample obtained in each experiment is shown (Table 1). The differences between the two experimental runs indicated that perhaps particle size of the dentin is an important factor in the yield of lipids obtained during extraction.

By means of thin-layer chromatography, it was possible to divide the lipids found in

* Silica Gel G, E. Merck, A. G., Darmstadt, Germany.

† Radin-Pelic, Applied Science Laboratories, University Park, Pa.

‡ Aerograph 1520, Aerograph Co., Union City, N.J.

dentin into: (a) hydrocarbons, (b) wax ester and sterol ester, (c) triglycerides, (d) free fatty acids, (e) free sterols, and (f) polar materials, which include monoglycerides, diglycerides, and phospholipids. The analytical values obtained for each of these fractions are shown (Table 2). The obtained Rf value for the steroid fraction indicates that in all probability this is free cholesterol.

TABLE 1

TOTAL LIPID CONTENT IN HUMAN DENTIN AS DETERMINED BY THIN-LAYER AND GAS-LIQUID CHROMATOGRAPHY

Experiment	Weight of Sample (Gm.)	Weight of Lipids Recovered (mg.)
1	100	94.3
2	100	86.5

The estimated analytical error is ± 10.5 percent

TABLE 2

COMPOSITION OF HUMAN DENTIN LIPID

Component	Weight/Percent of Lipid Exp. 1	Exp. 2
Observed Results		
Hydrocarbons	2.4	2.9
Wax esters and sterol esters	4.0	4.6
Triglycerides	26.0	23.0
Free fatty acids	50.0	47.0
Free sterols	3.4	6.4
Phospholipids	12.6	14.5
Corrected Results		
Hydrocarbons	2.4	2.9
Wax esters and sterol esters	5.2	6.0
Triglycerides	34.0	29.2
Free fatty acids	40.8	40.0
Free sterols	2.2	5.2
Phospholipids	12.6	14.5

The estimated analytical error was ± 7.5 percent for observed results. A 27 percent correction for hydrolysis of triglycerides and a 30 percent correction for sterol esters were applied to obtain the corrected results tabulated.

TABLE 3

FATTY ACID COMPOSITION OF HUMAN DENTIN LIPIDS

Fatty Acids	Exp. 1	Exp. 2
Saturated		
C:8–C:12	9.51	8.74
C:14	2.51	1.95
C:15	1.11	2.00
C:16	35.90	37.10
C:17	0.40	—
C:18	29.30	24.65
C:19	0.54	0.74
C:20	1.30	2.40
C:22	4.76	3.80
C:24	7.03	8.01
Unsaturated		
C:14:1	1.10	1.07
C:15:1	0.77	1.35
C:16:1	2.65	3.72
C:17:1	—	—
C:18:1	3.04	4.81

The estimated analytical error is ± 3.8 percent.

The fatty acid composition, obtained by gas-liquid chromatography of the methyl esters from the total saponification of the lipid sample, indicated a wide range of composition, with the saturated fatty acids prevailing and the amount of medium-chain fatty acid being slightly larger than expected. The actual chain length distribution of the long-chain fatty acid is not much different than the results that have been found for most tissues of the human being.

Discussion

The methods of preparation of the samples and extraction of the lipids caused denaturation and hydrolysis of the more complex fat components (steroid esters, triglycerides, and phospholipids). Free fatty acids would thus show higher levels than expected. There are, at present, no technics available that are capable of yielding all of the lipid components in dentin without causing hydrolysis of ester bonds. The knowledge of the extent of this hydrolysis permits an estimate of the original content. Once these corrections are taken into account, the numerical values obtained may be interpreted to indicate that no unusual distinct single lipid components or percentage lipid composition was present for dentin that is not otherwise found elsewhere in the body.[12,13]

Summary

A new method is described for the extraction of dentin lipids and preparation of the sample. Thin-layer chromatography was used to separate the lipids into six fractions. The chain length and saturation composition of all fatty acids found in dentin was determined by gas-liquid chromatography. Analytical data indicated that these methods and operations caused a minimum of 27 to 30 percent hydrolysis of the original esters. If these induced errors are taken into consideration, the results obtained indicate that the lipid composition of dentin is similar to the lipid composition found in most human tissues.

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